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Target site–based penoxsulam resistance in barnyardgrass (*Echinochloa crus-galli*) from China

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Research Article

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Abstract

Barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] is acknowledged to be the most troublesome weed in rice fields in Anhui and Jiangsu provinces of China. It cannot be effectively controlled using certain acetolactate synthase (ALS)-inhibiting herbicides, including penoxsulam. *Echinochloa crus-galli* samples with suspected resistance to penoxsulam were collected to identify the target site–based mechanism underlying this resistance. Populations AXXZ-2 and JNRG-2 showed 33- and 7.3-fold resistance to penoxsulam, respectively, compared with the susceptible JLGY-3 population. Cross-resistance to other ALS inhibitors was reported in AXXZ-2 but not in JNRG-2, and occasionally showed higher sensitivity than JLGY-3. In vitro ALS activity assays revealed that penoxsulam concentrations required to inhibit 50% of ALS activity were 11 and 5.2 times greater in AXXZ-2 and JNRG-2, respectively, than in JLGY-3. DNA and predicted amino acid sequence analyses of ALS revealed Ala-205-Val and Ala-122-Gly substitutions in AXXZ-2 and JNRG-2, respectively. Our results indicate that these substitutions in ALS are at least partially responsible for resistance to penoxsulam.

Introduction

Barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.], an allohexaploid grass species, is considered the most troublesome weed in rice (*Oryza sativa* L.) paddy fields, causing significant crop yield losses (Ni et al. 1996). At present, the application of herbicides is the most effective strategy for weed control, and herbicides with different sites of action, such as acetolactate synthase (ALS) and acetyl-coenzyme A carboxylase (ACCase) inhibitors, have been developed and widely used in rice fields to control *E. crus-galli*.

ALS, also known as acetohydroxyacid synthase, is the first enzyme in the biosynthesis pathway of three essential branched-chain amino acids, namely leucine, isoleucine, and valine (Duggleby et al. 2008; Ray 1984). It is the target site of many commercial herbicides, including sulfonylureas (SUs), imidazolinones (IMIs), pyrimidinylthiobenzoates (PTBs), triazolopyrimidines (TPs), and sulfonylaminocarbonyltriazolinones (SCTs) (Yu and Powles 2014). These herbicides are widely applied in weed control because of their low use rates, high efficiency, multicrop selectivity, broad spectrum of action, and season-long efficacy. Since 2008, penoxsulam, an ALS inhibitor, has been one of the most extensively used herbicides in rice fields in China; however, the continued use of penoxsulam has resulted in the rapid evolution of resistance in *E. crus-galli*. We have previously reported that, in certain locations in China, *E. crus-galli* developed resistance to penoxsulam in 2016 (Chen et al. 2016). This species, as well as other species of *Echinochloa*, is also reported to have developed resistance to penoxsulam in Turkey (Altop et al. 2014), the United States (Norsworthy et al. 2014), Italy (Panozzo et al. 2013), Greece (Kaloumenos et al. 2013), Japan (Iwakami et al. 2015), and South Korea (Song et al. 2017).

Mechanisms of herbicide resistance can be divided into target-site resistance (TSR) and non–target site resistance (Powles and Yu 2010). TSR is generally associated with relevant gene mutations, resulting in amino acid changes in a target enzyme that prevent or reduce herbicide binding (Yu and Powles 2014). With regard to ALS inhibitors, 28 amino acid substitutions at eight conserved positions in the *ALS* gene have been reported to date in various weed species (Tranel et al. 2018). These positions are Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653, and Gly-654 (numbered on the basis of the corresponding sequence of *Arabidopsis thaliana* L.) (Yu and Powles 2014). Substitutions at Ala-122, Ser-653, and Trp-574 have been

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reported to confer resistance to ALS-inhibiting herbicides in *E. crus-galli* and other *Echinochloa* species (Kaloumenos et al. 2013; Matzenbacher et al. 2015; Panozzo et al. 2013; Riar et al. 2013). Given that *E. crus-galli* is an allohexaploid grass species, multiple copies of genes encoding ALS may exist. For example, two complete *ALS* genes and the carboxyltransferase domain of four *ACCase* genes have been isolated in rice barnyardgrass [*Echinochloa phyllopogon* (Stapf) Koso-Pol.] (Iwakami et al. 2012), and the hexaploid wild oat (*Avena fatua* L.) has three *ACCase* gene copies, each associated with a different resistance mutation (Yu et al. 2013). These findings prompted us to look for multiple copies of the mutant target gene in *E. crus-galli*.

Cross-resistance patterns to ALS-inhibiting herbicides depend on the position and specificity of mutations. Mutations at Ala-122, Ala-205, Ser-653, and Gly-654 generally provide resistance to IMIs, whereas mutations at Pro-197 lead to resistance to SUs, and mutations at Trp-574 confer resistance to both SU and IMI herbicides (Tranel et al. 2018). Moreover, substitutions at Asp-376 and Arg-377 can result in cross-resistance to one or more IMI, PTB, SCT, SU, and TP herbicides (Tranel et al. 2018). The discovery of multiple mutations at the same position revealed that not only the position, but also the mutation type can affect cross-resistance patterns. For instance, at position 122, substitution of alanine with threonine causes resistance to IMI herbicides only, whereas substitution of alanine with tyrosine imparts a high level of resistance to IMI, SU, and TP herbicides (Han et al. 2012; Tranel et al. 2018), and substitution of alanine with valine confers partial resistance to some SU herbicides (Krysiak et al. 2011). Furthermore, the novel amino acid substitution Ala-205-Phe in annual bluegrass (*Poa annua* L.) confers broad-spectrum (IMI, PTB, SCT, SU, and TP) resistance to ALS-inhibiting herbicides (Brosnan et al. 2016). However, the patterns and levels of cross-resistance to ALS-inhibiting herbicides conferred by specific *ALS* mutations cannot be based on the response to one or two herbicides from a particular ALS-inhibitor chemical group (Han et al. 2012), that is, there is no absolute connection between cross-resistance patterns and specific *ALS* mutations.

Therefore, in the present study, we aimed to (1) identify *E. crus-galli* populations resistant to penoxsulam, (2) explore the target-site basis of this resistance, and (3) characterize the cross-resistance observed in some populations to provide information for improved management.

Materials and methods

Plant materials

In 2012, seeds of suspected resistant *E. crus-galli* populations (Table 1) were collected from rice fields in Jiangsu (JNRG-2) and Anhui (AXXZ-2) provinces (China), where the application of penoxsulam at the recommended dose during that year had failed to control this weed. Seeds of a susceptible population (JLGY-3) were collected from a recreational field that has never been exposed to herbicide treatment. All seeds were collected by hand, air-dried in the shade, and stored in paper bags at 4 C until use. For the purposes of the present study, we renamed these populations, which we have used in a previous study (Chen et al. 2016).

Table 1. Source locations of the three *Echinochloa crus-galli* populations examined in this study

Population	Location		Province
	Longitude	Latitude	
JNRG-2	116.92°E	32.28°N	Jiangsu
AXXZ-2	110.98°E	30.93°N	Anhui
JLGY-3	119.12°E	34.83°N	Jiangsu

Whole-plant dose response

Twenty seeds from each of the three populations were sown in plastic pots (9-cm diameter by 10-cm height) filled with a 2:1 (w/w) mixture of sand and pH 5.6 organic matter and planted in growth chamber at 30 C/25 C (light/dark temperature) with a 12-h light/12-h dark cycle, light intensity of 8,000 lx, and 85% relative humidity. Before herbicide treatment, seedlings were thinned to 12 plants pot^{-1}. At the 3- to 4-leaf stage, herbicides were applied using a laboratory sprayer (machine model: 3WP-2000, Nanjing Research Institute for Agricultural Mechanization, Nanjing, National Ministry of Agriculture of China) equipped with a flat-fan nozzle, delivering 280 L ha^{-1} at 230 kPa. On the basis of the findings of a preliminary experiment (unpublished data), penoxsulam was applied at 0, 3.75, 7.5, 15, 30, and 60 g ai ha^{-1} to the JNRG-2 population; at 0, 7.5, 15, 30, 60, and 120 g ai ha^{-1} to the AXXZ-2 population; and at 0, 0.94, 1.88, 3.75, 7.5, and 15 g ai ha^{-1} to the JLGY-3 population. Treated plants were returned to the incubators and cultured as described above. At 2 wk after penoxsulam application, the amount of fresh aboveground biomass was determined. This experiment was conducted twice in a completely randomized design with four replications.

Cross-resistance of the penoxsulam-resistant populations JNRG-2 and AXXZ-2 was determined using the methods described above. With the exceptions of imazapic, pyroxsulam, and flucarbazone-sodium, all of the ALS inhibitors selected for the bioassays are used in Chinese rice fields. Application doses were based on the results of a preliminary experiment (unpublished data) and are listed in Table 2. Other procedures were identical to those described above.

ALS activity assay

The response of the ALS enzyme to penoxsulam was determined using crude enzyme extracts. Seedlings at the 3- to 4-leaf stage from JNRG-2, AXXZ-2, and JLGY-3 were used for in vitro assays of ALS activity as described by Yu et al. (2004), with slight modifications as follows. Leaf blades were harvested from each population (3 g), powdered in liquid nitrogen, and suspended in enzyme extraction buffer (4.5 ml) containing 100 mM potassium phosphate buffer (pH 7.5), 10 mM sodium pyruvate, 1 mM $MgCl_2$, 1 mM thiamine pyrophosphate, and 10 mM flavine adenine dinucleotide (FAD). Each reaction contained 100 μl of protein extract, 200 μl of enzyme assay buffer (100 mM potassium phosphate buffer pH 7.5, 200 mM sodium pyruvate, 20 mM $MgCl_2$, 2 mM thiamine pyrophosphate, 20 μM FAD, 1 mM dithiothreitol), and 100 μl of ALS inhibitor (penoxsulam at 0.005, 0.05, 0.5, 5, and 50 μM). A nontreated (no herbicide applied) control was included in each assay for comparison. Acetoin was formed by incubation at 37 C for 60 min. The reaction was stopped by the addition of 8 μl of 6 N H_2SO_4, and the mixture was maintained at 60 C for 30 min before the addition of 100 μl of creatine solution (0.55%) and 100 μl of α-naphthol solution (5.5% in 5 N NaOH). ALS activity was

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Table 2. Herbicide doses applied in dose–response tests

Group[a]	Herbicide[b]	Recommended field dose —g ai ha^{-1}—	Doses applied to each population —g ai ha^{-1}—		
			JNRG-2	AXXZ-2	JLGY-3
PTB	Pyribenzoxim	37.5	0,2.35,4.69,9.38,18.75,37.5	0,4.69,9.38,18.75,37.5,75	0,2.35,4.69,9.38,18.75,37.5
IMI	Imazapic	108	0,3.38,6.75,13.5,27,54	0,6.75,13.5,27,54,108	0,3.38,6.75,13.5,27,54
SCT	Flucarbazone-sodium	30	0,1.88,3.75,7.5,15,30	0,3.75,7.5,15,30,60	0,1.88,3.75,7.5,15,30
TP	Penoxsulam	30	0,3.75,7.5,15,30,60	0, 7.5,15,30,60,120	0,0.94,1.88,3.75,7.5,15
	Pyroxsulam	14	0,0.88,1.75,3.5,7,14	0,1.75,3.5,7,14,28	0,0.88,1.75,3.5,7,14
SU	Flucetosulfuron	30	0,1.88,3.75,7.5,15,30	0,3.75,7.5,15,30,60	0,1.88,3.75,7.5,15,30
	Propyrisulfuron	82.5	0,5.41,10.81,20.63,41.25,82.5	0,10.81,20.63,41.25,82.5,165	0,5.41,10.81,20.63,41.25, 82.5
	Rimsulfuron	22.5	0,1.41,2.81,5.63,11.25,22.5	0,2.81,5.63,11.25,22.5,45	0,1.41,2.81,5.63,11.25,22.5,

[a]Abbreviations: IMI, imidazolinone; PTB, pyrimidinylthiobenzoate; SCT, sulfonylaminocarbonyltriazolinone; SU, sulfonylurea; TP, triazolopyrimidine.
[b]Pyribenzoxim (5% emulsifiable concentrate; Korea's LG Life Science Co. Ltd., Shanghai, China); imazapic (240 g L^{-1} aqueous suspension; Rotam, Suzhou, China); flucarbazone-sodium (70% water-dispersible granules [WDG]; Arysta LifeScience, Shanghai, China]; pyroxsulam (7.5% WDG; Dow AgroSciences, Beijing, China); penoxsulam (25g L^{-1} oil dispersion; Dow AgroSciences, Beijing, China); flucetosulfuron (10% wettable powder; FMC, Suzhou, China); propyrisulfuron (100 g L^{-1} suspension concentrate; Sumitomo Chemical Co. Ltd., Japan); rimsulfuron (25% WDG; Jiangsu Futian Agrochemical Co., Ltd., China).

monitored colorimetrically (530 nm) using a microplate photometer (Thermo Fisher, Waltham, MA, USA) by measuring acetoin production (pure acetoin was used as the standard). The concentration of protein in the extracts was measured using the Bradford method (Bradford 1976), with bovine serum albumin used as a standard. The assay was performed twice with independent extracts, each with three replications per concentration.

Gene cloning and sequencing

Young shoot tissues obtained from individual plants at the 3- to 4-leaf stage were used for DNA extraction using a Plant Genomic DNA kit (Tiangen Biotech, Beijing, China), according to the manufacturer's instructions. A specific pair of primers (forward: TTGCCACCCTCCCCAAACCC; reverse: GCACCAC TCGCTGAAATCCG) was designed using Primer Premier v. 5.0 (PREMIER Biosoft International, Palo Alto, CA, USA) based on the *ALS* gene sequences of *Echinochloa crus-galli* var. *crus-galli* (accession LC006061.1) and *Echinochloa crus-galli* var. *formosensis* (accession LC006063.1), retrieved from the National Center for Biotechnology Information (NCBI) GenBank database, and was used to amplify the complete sequence of *ALS* in *E. crus-galli*. The polymerase chain reaction (PCR) mixture contained 2.5 ng of template DNA, 2 µl of each primer (10 µM), 25 µl of 2× Phanta Max Master Mix (Vazyme Biotech, Nanjing, China), and ddH_2O to a final volume of 50 µl. Amplification was conducted as follows: 5 min at 95 C for DNA denaturation; 35 cycles of 30 s at 95 C for DNA denaturation, 30 s at 58 C for annealing, and 90 s at 72 C for DNA elongation; and a final elongation for 7 min at 72 C. The PCR products were purified using a TaKaRa MiniBEST agarose gel DNA extraction kit (TaKaRa Biotechnology, Dalian, China). After addition of poly(A) using the TaKaRa Taq Kit (TaKaRa Biotechnology), the resulting productions were cloned into a pMD19-T vector (TaKaRa Biotechnology). Plasmids containing the fragment insertion were bidirectionally sequenced by GenScript Biotechnology (Nanjing, China). Ten plants from each of the three populations were selected for gene cloning. At least 12 transformed clones of each plant were sequenced to obtain complete *ALS* sequences, which were aligned and compared using BioEdit Sequence Alignment Editor v. 7.2.5 (Tom Hall, Carlsbad, CA, USA). The Basic Local Alignment Search Tool (BLAST) procedure within the NCBI database was used to verify the accuracy of the obtained sequences.

Table 3. Sensitivity of the three *Echinochloa crus-galli* populations to penoxsulam and other acetolactate synthase inhibitors

Herbicide	Population	ED_{50}[a]	RI[b]
Penoxsulam	JNRG-2	65.33	33.33
	AXXZ-2	14.34	7.32
	JLGY-3	1.96	
Pyribenzoxim	JNRG-2	9.50	1.00
	AXXZ-2	73.05	7.64
	JLGY-3	9.56	
Imazapic	JNRG-2	2.39	0.32
	AXXZ-2	20.36	2.68
	JLGY-3	7.58	
Flucarbazone-sodium	JNRG-2	1.06	0.16
	AXXZ-2	61.02	8.97
	JLGY-3	6.80	
Pyroxsulam	JNRG-2	2.23	0.85
	AXXZ-2	26.39	10.07
	JLGY-3	2.62	
Flucetosulfuron	JNRG-2	2.18	0.80
	AXXZ-2	44.38	16.37
	JLGY-3	2.71	
Propyrisulfuron	JNRG-2	5.33	0.97
	AXXZ-2	59.24	10.79
	JLGY-3	5.49	
Rimsulfuron	JNRG-2	2.69	1.00
	AXXZ-2	22.44	8.34
	JLGY-3	2.69	

[a]ED_{50} refers to the effective dose of herbicide causing 50% inhibition of fresh weight and is indicated as grams of active ingredient per hectare (g ai ha^{-1}).
[b]RI is the resistance index. Herbicide resistance was classified into five groups: no resistance (RI < 2); low resistance (RI = 2–5); moderate resistance (RI = 6–10); high resistance (RI = 11–100); and very high resistance (RI >100).

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Table 4. The single-nucleotide polymorphisms in multiple sites of acetolactate synthase sequences in the *E. crus-galli* populations examined

	Single-nucleotide polymorphisms							
	235 Position		283 Position		318 Position		441 Position	
Sequence name	Codon	Amino acid	Codon	Amino acid	Codon	Amino acid	Codon	Amino acid
JLGY-3 ALS1;2	ATA	Met	CCT	Pro	CGC	Arg	CAA	Gln
JLGY-3 ALS2;2	GTG	Val	CCT	Pro	TGC	Cys	CAC	His
JNRG-2 ALS1;2	ATA	Met	CCT	Pro	CGC	Arg	CAA	Gln
JNRG-2 ALS2;2	GTG	Val	CCT	Pro	TGC	Cys	CAC	His
AXXZ-2 ALS1;3	ATA	Met	CAT	His	CGC	Arg	CAA	Gln
AXXZ-2 ALS2;3	GTG	Val	CAT	His	CGC	Arg	CAA	Gln
AXXZ-2 ALS3; 3	GTG	Val	CCT	Pro	TGC	Cys	CAC	His

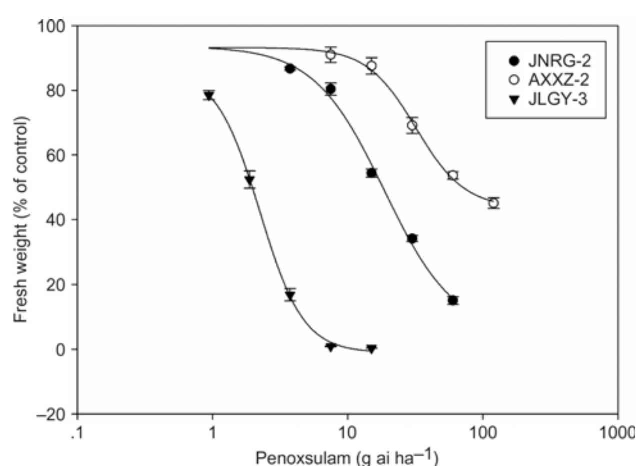

Figure 1. Fresh weight of the aboveground parts of three populations of *Echinochloa crus-galli* treated with penoxsulam. Vertical bars represent the mean ± SE.

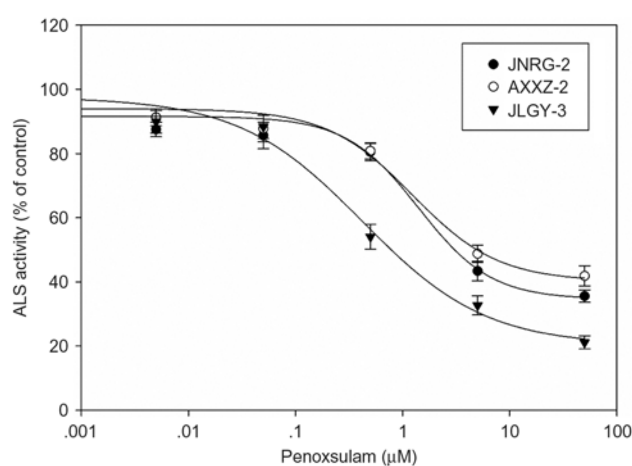

Figure 2. In vitro acetolactate synthase (ALS) activity of three *Echinochloa crus-galli* populations when treated with penoxsulam. Vertical bars represent the mean ± SE.

Data analysis

Whole-plant dose–response data were subjected to ANOVA using SPSS v. 21.0 (IBM, Armonk, NY). The ANOVA results showed no significant difference between assay repetitions, so the results for the repeat assays were averaged. Data were then pooled and fit to the four-parameter nonlinear logistic-regression model (Equation 1), calculated using SigmaPlot v. 10.0 (SigmaPlot Software, Chicago, IL, USA), to determine the effective dose of herbicide causing 50% inhibition of fresh weight (ED_{50}):

$$Y = \frac{c + (d - c)}{\left[1 + \left(\frac{x}{g}\right)^{b}\right]} \qquad [1]$$

where Y denotes fresh weight, expressed as a percentage of the nontreated control at dose x of the herbicide; b is the slope; c is the lower limit; d is the upper limit; and g is the herbicide dose at the point of inflection, halfway between the upper and lower limits (Xu et al. 2014).

The same analysis was used to calculate the herbicide concentrations required to inhibit 50% of ALS activity (IC_{50}) in enzymatic assays. Resistance indexes (RIs) were calculated by dividing the ED_{50} (or IC_{50}) of the resistant population (R) by the ED_{50} (or IC_{50}) of the susceptible population (S).

Results and discussion

Sensitivity to penoxsulam

The ED_{50} of AXXZ-2 (65.33 g ha^{-1}) was considerably higher than the recommended application dose (15 to 30 g ha^{-1}), whereas those of JNRG-2 (14.34 g ha^{-1}) and JLGY-3 (1.96 g ha^{-1}) were lower than the recommended dose (Table 3; Figure 1). The RIs of the resistant populations AXXZ-2 and JNRG-2 were 33 and 7.3, respectively, indicating a high and moderate resistance, respectively, to penoxsulam, according to Beckie and Tardif (2012).

Gene cloning and sequence analysis

To our knowledge, this is the first study reporting the complete coding sequence (CDS) of *ALS* in *E. crus-galli* obtained using a single pair of primers. The difficulty in obtaining the complete CDS of *ALS* is related to the extremely high GC content in its 5′ region. Two *ALS* sequences of JNRG-2 and JLGY-3 and three *ALS* sequences of AXXZ-2 were isolated, and the mutant sequences were submitted to the NCBI database (GenBank accession numbers MH013500 and MH013488). The BLAST procedure conducted for *ALS* sequences revealed that these were highly similar (96% to 97%) to the reported sequences LC006061.1 (see Supplementary Table S1), indicating that we had amplified the correct *ALS* sequences from *E. crus-galli*.

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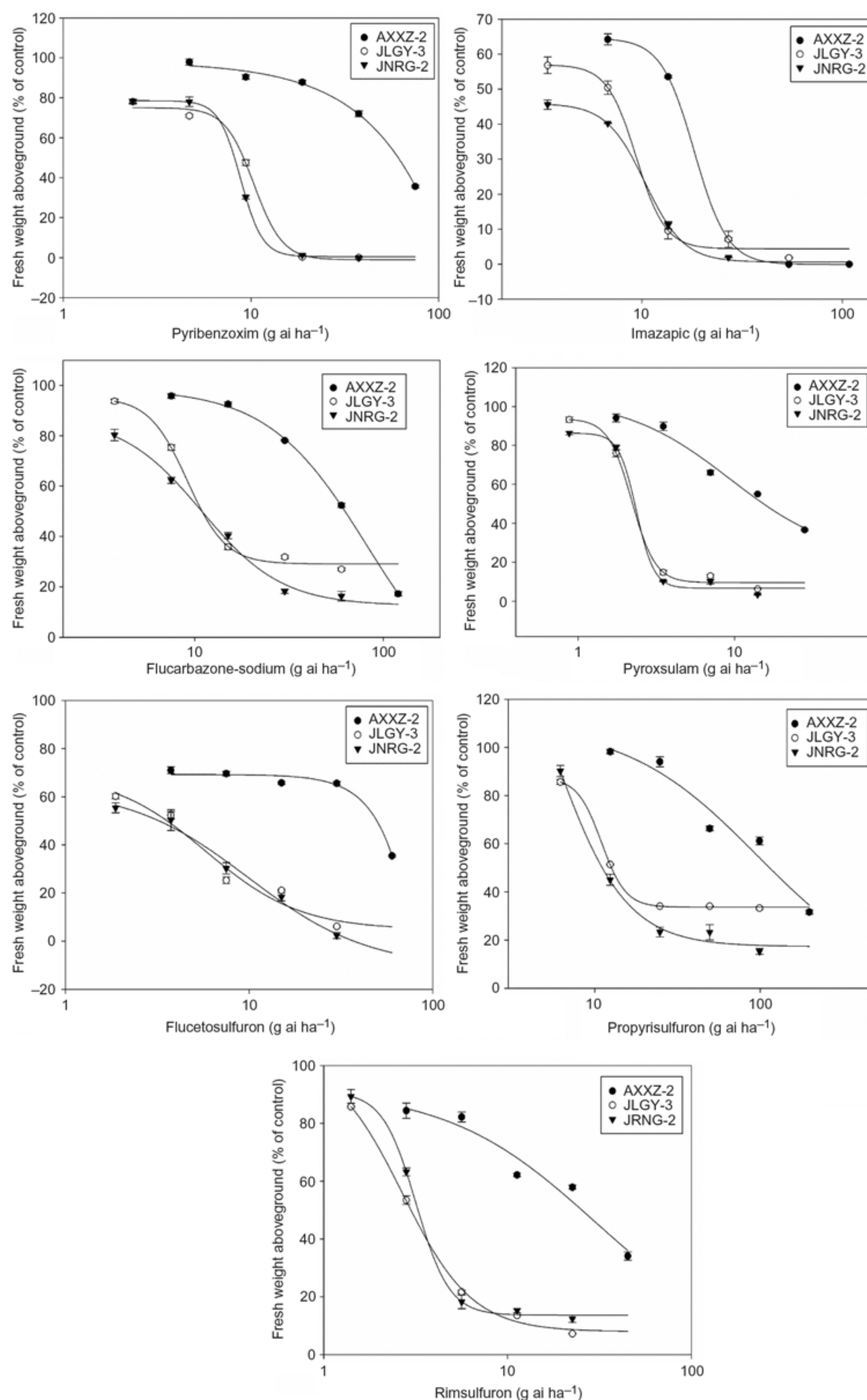

Figure 3. Fresh weight of the aboveground parts of three populations of *Echinochloa crus-galli* treated with seven acetolactate synthase (ALS)-inhibiting herbicides. Vertical bars represent the mean ± SE.

Thus, the primer pair designed for the present study might be useful for acquiring the complete sequences of *ALS* in other *Echinochloa* species. After further analysis of the obtained sequences, we identified single-nucleotide polymorphisms at multiple conserved sites between different copies (Table 4). Based on the amino acid residues at these positions, we can determine the copy numbers and infer the sequence of the *ALS* copy in *E. crus-galli*. The variation in *ALS* gene copy number observed in the present study is consistent with that previously reported in shortawn foxtail (*Alopecurus aequalis* Sobol.)

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(Iwakami et al. 2017) and Japanese foxtail (*Alopecurus japonicus* Steudel) (Feng et al. 2017).

A nucleotide mutation (GCC to GGC) was found in the *ALS2;2* sequence of JNRG-2 when compared with the corresponding sequence in JLGY-3, which results in the substitution of alanine with glycine at position 122. Another nucleotide mutation (GCC to GTC) was detected in the *ALS1;3* sequence of AXXZ-2, which results in the substitution of alanine with valine at position 205 (positions are numbered relative to the *ALS* of *A. thaliana*). However, none of the mutants known to confer resistance to ALS inhibitors in *E. crus-galli* or other *Echinochloa* species, namely Trp-574 and Ser-653 (Kaloumenos et al. 2013; Matzenbacher et al. 2015; Panozzo et al. 2013), were detected in the present study. Target-gene mutation is responsible for most resistance to ALS-inhibiting herbicides (Yu and Powles 2014). The Ala-205-Val substitution was documented for the first time in *E. crus-galli*, although previous studies have revealed that this mutation is the target-site basis for the resistance to ALS-inhibiting herbicides in eastern black nightshade (*Solanum ptychanthum* Dunal) (Ashigh and Tardif 2017), *Erigeron* spp. (Matzrafi et al. 2015), and redroot pigweed (*Amaranthus retroflexus* L.) (McNaughton et al. 2005). Amino acid mutations at position 122 have previously been reported to confer resistance to ALS inhibitors (Panozzo et al. 2017; Riar et al. 2013). In summary, our findings indicate that the amino acid mutations detected in the two penoxsulam-resistant populations are the main target-site basis for penoxsulam resistance in *E. crus-galli*.

In vitro ALS inhibition assays

The inhibitory effect of penoxsulam on ALS activity was observed to be less pronounced in AXXZ-2 and JNRG-2 than in JLGY-3 (Figure 2). The IC_{50} values determined for AXX-2 and JNRG-2 were 13.02 and 5.89 μM, respectively, which were 11- and 5.2-fold greater than that for JLGY-3 (1.14 μM), suggesting that the low sensitivity of ALS occurs in conjunction with resistance to penoxsulam in *E. crus-galli* populations. In addition, this trend was consistent with our whole-plant dose–response analysis, although the RIs were not high compared with the whole-plant dose response. These results strongly support the hypothesis that a TSR mechanism is responsible for the resistance of *E. crus-galli* populations.

Sensitivity to other ALS inhibitors

On the basis of ED_{50} and RI values, we examined cross-resistance patterns in the resistant populations of *E. crus-galli*. Cross-resistance to ALS inhibitors was found in AXXZ-2 (Table 3; Figure 3), which was resistant to IMI, PTB, SCT, TP, and SU herbicides, whereas JNRG-2 was sensitive to other ALS inhibitors, and sometimes even more sensitive than JLGY-3, which was the susceptible population in the present study. With regard to the ALS Ala-122-Gly mutation found in the JNRG-2 population, we conjecture that there might be a negative interaction between the Ala-122-Gly mutation and other ALS inhibitors. Negative cross-resistance is not rare and has been reported in yeast (Duggleby et al. 2003) and tobacco (*Nicotiana tabacum* L.) (Le et al. 2005). An Asp-376-Asn substitution in yeast ALS and an Asp-376-Ala substitution in tobacco ALS conferred higher resistance to SU herbicides, but rendered plants more sensitive to IMI herbicides. In the present study, we also observed that, in response to penoxsulam treatment at 30 g ha^{-1}, the growth of the resistant population bearing the Ala-122-Gly mutation was strongly inhibited compared with that of the population harboring the Ala-205-Val substitution. This is similar to the response observed in resistant wild radish (*Raphanus raphanistrum* L.) homozygous for the Asp-376-Gln mutation and other mutations affecting plant growth (Yu and Powles 2014). Given the cross-resistance patterns and plant growth, the *ALS* Ala-122 mutation found in resistant populations of *E. crus-galli* appears to be a TSR mechanism and similar to Asp-376.

In the present study, the AXXZ-2 population exhibited cross-resistance to five classes of ALS inhibitors, which might be due to the Ala-205-Val substitution. The growth of treated AXXZ-2 seedlings was virtually uninhibited by penoxsulam at 30 g. ha^{-1} and was not notably different from that of untreated seedlings. In addition, the AXXZ-2 population was resistant to IMI, PTB, SCT, TP, and SU herbicides, with RI values varying from 2.68 to 16.37. These observations indicated that AXXZ-2 showed different levels of resistance to other ALS inhibitors, ranging from low to high. Interestingly, the AXXZ-2 population was resistant to four herbicides to which it had never previously been exposed (pyroxsulam and flucarbazone-sodium are only used in wheat (*Triticum aestivum* L.) fields, imazapic has not been used in rice fields in China, and propyrisulfuron is a novel herbicide registered in China in 2015), which presents a considerable challenge in terms of weed control and management. The Ala-205 mutation has been reported in several weeds, but its cross-resistance pattern is less clear than those associated with Pro-197 and Trp-574. Overall, this mutation could cause resistance to IMI herbicides. Recently, an amino acid substitution (Ala-205-Phe) in ALS was shown to confer broad-spectrum resistance to ALS-inhibiting herbicides in *P. annua* (Brosnan et al. 2016), which in certain respects is consistent with the results of the present study.

Conclusions

In the *E. crus-galli* populations we studied, AXXZ-2 and JNRG-2 had evolved different levels of penoxsulam resistance confirmed by whole-plant dose response. After analysis of the *ALS* sequences of single plants in resistant populations, two novel mutations (Ala-122-Gly and Ala-205-Val) were detected. To our best knowledge, this is the first time these two mutations have been reported in resistant *Echinochloa* species, although the Ala-122-Val and Ala-122-Thr substitutions had been revealed in *E. crus-galli* resistant to ALS-inhibiting herbicides (Riar et al. 2013). Lower sensitivity of ALS to penoxsulam was also revealed in the two resistant populations compared with the susceptible population. Overall, amino acid substitutions and the low sensitivity of ALS to penoxsulam might be the target-site bases for resistance to penoxsulam in *E. crus-galli*, as they are the typical target-site bases of resistance to ALS inhibitors in many other *Echinochloa* species. Moreover, the AXXZ-2 population was resistant to all ALS inhibitors tested, which might cause great difficulty in controlling this biotype, whereas JNRG-2 was not. The cross-resistance patterns in resistant populations harboring different mutations reported here highlight the need to apply herbicides scientifically and to effectively to manage *E. crus-galli* in rice fields.

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